Isoelectric Focusing: A Novel Approach for Isolation of Small, Water-Soluble Molecules from Natural Products Extracts

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INTRODUCTION

Few chromatographic techniques are applicable to those very polar molecules found in water soluble extracts. Ion exchange and size exclusion chromatography have yielded some successes, but isoelectric focusing has not been applied to this separation dilemma.

Isoelectric focusing, long used in protein purification, is a technique wherein a mixture of variously charged water-soluble molecules (called ampholytes) migrate to their isoelectric points (pI) in an electrical field, thus establishing a pH gradient. Non-ampholyte charged molecules in this system will migrate along the pH gradient until the net charge on the molecule is zero. We have investigated isoelectric focusing for its applicability as a new chromatographic tool useful in natural products chemistry for resolving the small, bioactive, water-soluble molecules in crude water-soluble extracts.

MATERIALS AND METHODS

Equipment:

- Biorad ROTOFOR Isoelectric Focusing apparatus with 60-ml chamber
- Ampholyte - pH range 3-10 (3 ml per run)
- Pharmacia Electrophoresis Constant Power Supply (3000V/15W)
- Orion Model 250 A pH meter
- Orion Model 900 A printer
- Beckman Model 511084 epoxy probe
- Ion-exchange resin (molecular biology grade AG501- X8 D)

Sample Preparation:

- Extract of higher plant stem and twigs - chosen for toxicity against *Candida albicans*
- Sample dissolved in RT Milli-Q water/acetonitrile (3:1) and bound to bulk C18 packing
- Eluted with a water/acetonitrile gradient
- Toxic fractions combined and passed down a Sephadex G25 column
- Toxic fractions resolubilized and mixed with ampholyte
- Sample placed on the ROTOFOR and processed ~5 hours 20 fractions collected and pH of each recorded

Toxicity Assays:

- Fractions were placed into wells of microtiter plates at 50 ug/well and dried
- Test organisms in growth media were added to each well
- Plates were incubated for 18-96 hrs
- Optical densities were read at 450 or 650 nm and percent growth inhibition (% GI) was calculated

EXPERIMENTAL

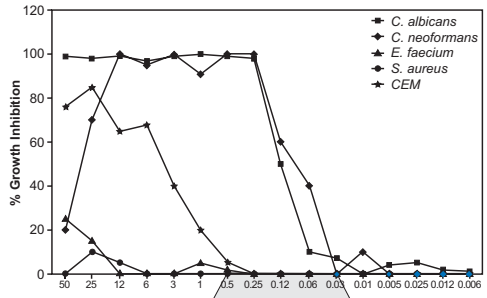
- Since the presence of ampholytes in the bioassays could potentially change the toxicity profile, the following control experiments were performed:

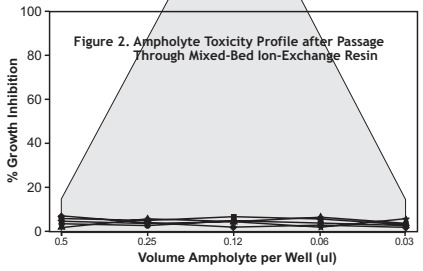
• The ampholyte mixture was serially diluted and bioassayed to quantify toxicity. Additionally, following isoelectric focusing, each fraction along the pH gradient was separately bioassayed, as certain components of the ampholyte mixture, concentrated into a narrow pH zone, could prove more toxic than the original mixture. Data (not shown) indicated that there was no zone of greater toxicity.
• A mixed-bed ion-exchange resin (AG501) has been shown to remove ampholytes from aqueous solutions. After determining the toxicity profile of the ampholyte, five fractions bracketing the %GI50 point were percolated through a resin bed and retested.
• An aliquot of the extract was passed through the ion-exchange resin and the eluate bioassayed. Toxicity was recovered in the eluate for the extract.

Figure 1 shows that there is a variable response from the five bioassay systems to the ampholyte. *C. albicans* and *C. neoformans* are highly sensitive to the presence of ampholytes, while *E. faecium* and *S. aureus* are unaffected. Passage of the ampholyte solution through a short column containing the mixed-bed resin AG501 effectively removes the ampholytes, as demonstrated by the lack of toxicity, shown in Figure 2.

- Since it was shown that ampholytes are toxic in some bioassay systems, the fractions from the ROTOFOR separations were divided into two aliquots, one tested as-is, and the other passed through the mixed-bed ion-exchange resin. After recording the pH of the processed fractions, both were transferred to microtiter plates for testing against *C. albicans*, *C. neoformans*, *E. faecium*, *S. aureus*, and CEM/SS (a human lymphocyte cell line).

Figure 1. Ampholyte Serial Dilution Toxicity Profile





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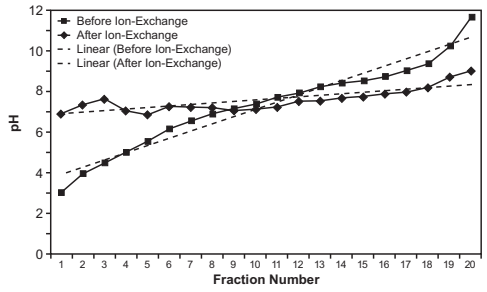
RESULTS

The 'flattening' of the pH profile, shown by Figure 3, demonstrates that passage of each fraction through a mixed-bed resin has removed the ampholytes, as each processed fraction has a pH of ~7.

Figure 3. Aqueous Plant Extract - pH Comparison



In Figure 4, the toxicity of fractions to *C. albicans* both before and after mixed-bed resin processing is shown. While the presence of the ampholyte has essentially no effect on toxicity as determined by CEM (Figure 5), the presence of a toxic component between wells ~11-17 would be masked by ampholyte toxicity when *C. albicans* is the test organism. A concentrated band of toxicity is found in three fractions in the 9-11 pH range. Previous attempts at isolating the toxic compounds from this terrestrial plant extract by standard column chromatographic methods resulted in toxicity being lost. Liquid/liquid partitioning also failed to resolve the active component.

An intensely blue fermentation broth from a fungus of unknown Genus/species was examined to determine whether the color could be resolved into multiple bands. As is seen in Figure 6, a number of compounds are present.

Figure 4. Plant Extract Toxicity Profile Against *C. albicans* Before and After Ampholyte Removal

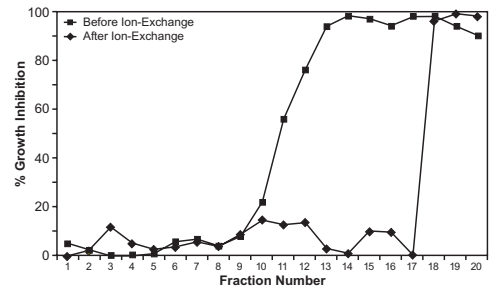


Figure 5. Ampholyte Toxicity Profile Against CEM/SS Cell Line

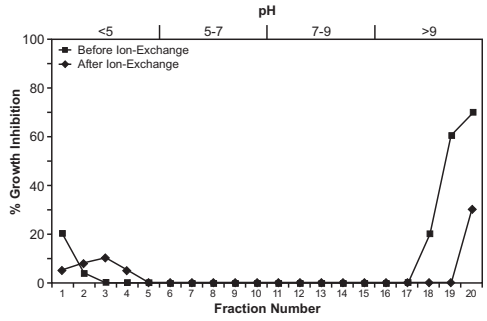
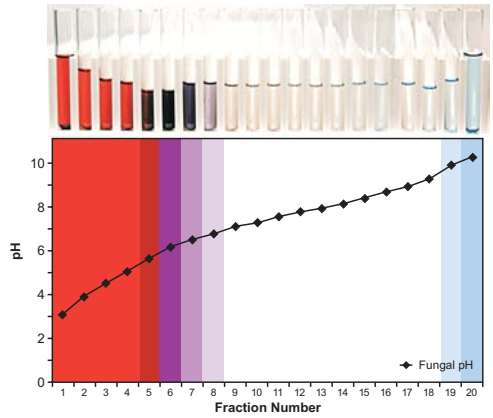


Figure 6. Aqueous Fungal Extract pH/Color Indication



CONCLUSION

We were able to use the Biorad ROTOFOR isoelectric focusing apparatus to concentrate and partially isolate the active components from various aqueous extracts. The method has been used successfully on additional marine, fungal, and terrestrial plant extracts with promising results. Test results from the above assay showed localized zones of activity for many of the compounds. The passage of the material through the ion-exchange resin bed effectively removed the ampholyte from the samples, while the toxicity of the parent sample was retained. The twig and bark extract provided a good example of the ability of the ROTOFOR system to handle problematic isolations. While the ampholytes may cause false positive hits in certain screens, their removal is easily achieved.

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